The GTPase Rap1 controls functional activation of macrophage integrin αMβ2 by LPS and other inflammatory mediators

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Background: β2 integrins mediate many aspects of the inflammatory and immune responses, including adhesion of leukocytes to the endothelium, complement-mediated phagocytosis in macrophages and neutrophils, and antigen-specific conjugate formation between cytotoxic T cells and their targets. A variety of inflammatory mediators, such as tumor necrosis factor-α (TNF-α), platelet-activating factor (PAF), and lipopolysaccharide (LPS) and other bacterial products induce the functional activation of β2 integrins, but the signaling events that link membrane receptors to integrin activation are poorly understood.

Results: We report here that expression of the constitutively active small GTPases Rap1 or R-ras, but not Ras or RalA, is sufficient for functional activation of αMβ2, the complement receptor 3 (CR3), in macrophages, allowing phagocytosis of C3bi-opsonized targets. Inhibition of Rap1, but not other Ras-like or Rho-like small GTPases, abolishes activation of αMβ2 induced by phorbol esters, LPS, TNF-α or PAF. Finally, Rap1 activation specifically controls the binding properties of αMβ2 towards its physiological ligand, namely the complement-opsonized phagocytic targets.

Conclusions: In macrophages, the Rap1 GTPase regulates activation of the αMβ2 integrin in response to a wide variety of inflammatory mediators.

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Background

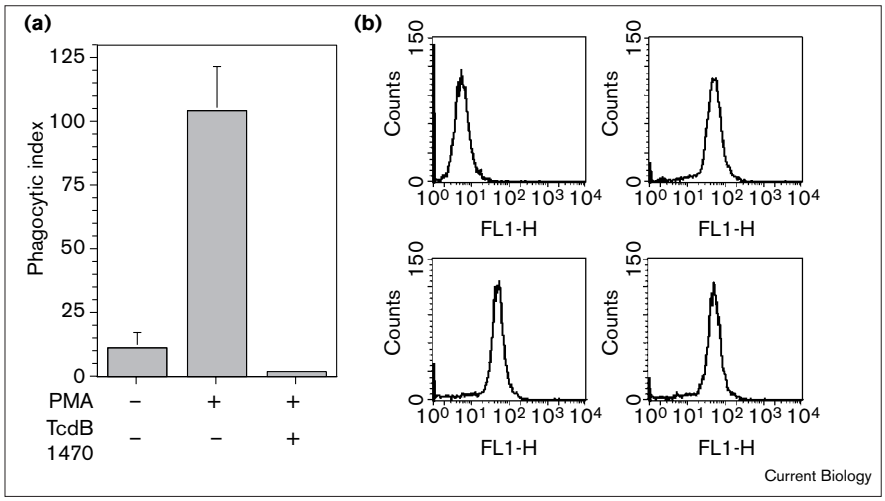
Leukocytes, which are responsible for mediating inflammation and immunity, remain inactive and non-adherent while circulating in the blood, but can be rapidly activated and recruited to tissues in response to inflammatory mediators or infection. Integrins of the β2 subfamily are required for leukocyte adhesion to endothelial cells and migration into tissues; they also mediate effector functions, such as the interaction of natural killer (NK) and cytotoxic T cells with their targets, the binding of T cells to antigen-presenting cells and the phagocytosis of complement-opsonized particles [1,2]. This crucial role of β2 integrins is most clearly demonstrated in patients with leukocyte adhesion deficiency (LAD) syndrome, where mutations in this integrin chain (CD18) dramatically increase susceptibility to bacterial infections [3]. Functional activation of the three major β2-containing integrins, αLβ2 (CD11a/CD18, LFA-1), αMβ2 (CD11b/CD18, CR3), and αXβ2 (CD11c/CD18, p150,95), is induced in several pro-inflammatory conditions, for example after T-cell receptor crosslinking and in response to a variety of inflammatory mediators such as lipopolysaccharide (LPS) and other bacterial products, tumor necrosis factor-α (TNF-α) and platelet-activating factor (PAF) [1,4–9]. The signal transduction pathway linking the membrane receptors for these diverse agonists to β2 integrin activation is not clear. As there have been several reports that small GTPases of the Ras family can regulate integrin function in a variety of cell types [10–14] we have examined their role in the activation of the αMβ2 complement receptor in the macrophage. We show that the Rap1 GTPase specifically regulates functional activation of the αMβ2 integrin in response to a wide variety of inflammatory mediators.

Results and discussion

To determine whether Ras-like small GTPases are involved in activation of the αMβ2 CR3 receptor, we first made use of TcdB-1470, a *Clostridium difficile* toxin that has been reported to glucosylate and inactivate Rac and also three members of the Ras family — R-ras, RalA and Rap1 [15]. As shown in Figure 1a, TcdB-1470 completely abolished (at least 95% inhibition) the phagocytosis of C3bi-opsonized sheep red blood cells in J774.A1 murine macrophage cells stimulated with the phorbol ester PMA. Inhibition of phagocytosis by TcdB-1470 was concentration-dependent and did not affect cell viability (data not shown). Using flow cytometry, we confirmed that PMA (and TcdB-1470) does not affect the surface expression of αMβ2 (Figure 1b).

As we have previously shown that Rac is not involved in CR3-mediated phagocytosis [16], the toxin result suggests that the three Ras-like GTPases, R-ras, RalA or Rap1 might be involved in PMA-induced αMβ2 activation. To

Figure 1



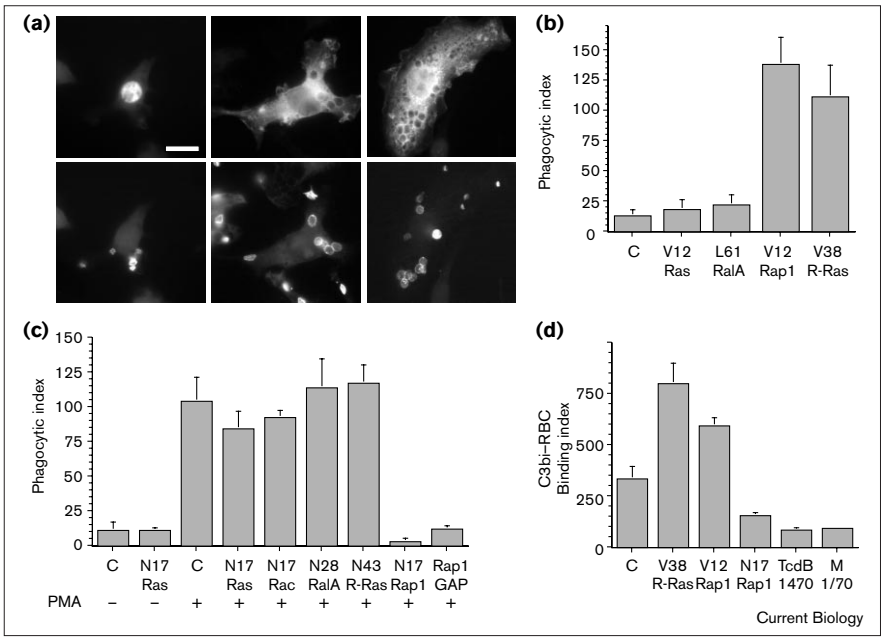
A Ras-like GTPase controls αMβ2 function. **(a)** TcdB-1470 (5 pg/ml) abolishes PMA-induced CR3-mediated phagocytosis in J774.A1 macrophages (mean ± SEM of three independent experiments). **(b)** Activation of αMβ2, or its inactivation by TcdB-1470, are independent of changes in surface expression of αM, as shown by flow cytometry. Top left, isotype control; top right, resting cells; bottom panels, PMA-stimulated cells preincubated with medium (left) or TcdB-1470 (5 pg/ml; right). Results are representative of three independent experiments. FLH-1, fluorescence intensity in arbitrary units.

determine whether Ras-like small GTPases can activate αMβ2, expression constructs encoding constitutively activated versions of R-ras (V38), RalA (L72), Rap1 (V12) and Ras (V12) were microinjected into macrophage cells and phagocytosis of C3bi-opsonized particles determined. Figure 2a shows that expression of either R-Ras (V38) or Rap1 (V12) was sufficient to trigger efficient CR3-mediated phagocytosis. Both the phagocytic index and the percentage of cells able to phagocytose C3bi-opsonized RBC increased dramatically upon macrophage activation or expression of active R-Ras or Rap1 (control, 9 ± 4%; PMA-activated cells, 69 ± 10%; R-Ras(V38)-expressing cells, 67 ± 4%; Rap1(V12)-expressing cells, 68 ± 11%; $n = 3$). Control cells never show more than one internalized particle per cell, whereas >50% of R-ras, Rap1-expressing and PMA-activated cells show between two and seven phagocytosed particles per cell. By contrast, RalA (L72) or Ras (V12) did not induce β2-dependent phagocytosis (Figure 2b). We conclude that when overexpressed, two members of the Ras family, R-Ras and Rap1, can induce functional activation of the αMβ2 integrin in macrophages.

To identify which of these GTPases actually mediates integrin activation in macrophages, we first microinjected J774.A1 cells with expression constructs encoding dominant-negative versions of R-ras and Rap1 and then activated the cells with PMA. As shown in Figure 2c, dominant-negative Rap1 (N17) completely inhibited phagocytosis to below control levels, whereas dominant negative R-Ras (N43), Rac (N17) and RalA (N28) had no effect on CR3-mediated phagocytosis of C3bi-opsonized targets. To confirm the specificity of the dominant-negative Rap1, a construct encoding a Rap-specific GTPase-activating protein, Rap1GAP [17], was microinjected into macrophages and this also completely inhibited PMA-induced activation of αMβ2 and phagocytosis (Figure 2c). In a further control, we have shown that expression of Rap1 (N17) does not affect the ability of J774 cells to phagocytose IgG-opsonized targets (data not shown), a process known to be independent of macrophage activation [18]. Ras has been reported to downregulate integrin function in some cell types [13,19]. To determine whether Ras exerts a tonic inhibitory effect on αMβ2 in macrophages, we injected a dominant-negative Ras (N17) construct. This did not lead to activation of CR3 function nor did it inhibit PMA-induced activation (Figure 2c).

To determine whether Rap1 regulates the ability of αMβ2 to interact with complement-opsonized targets, or the ability of αMβ2 integrin to internalize bound particles, we measured the effect of modifying Rap1 on particle binding. Figure 2d shows that expression of active Rap1 or R-Ras induces a two- to threefold increase in the number of C3bi-opsonized RBC bound by macrophages. In non-activated J774 cells, inhibition of Rap1, either through expression of the dominant-negative Rap1 or after treatment with TcdB-1470, reduced binding to around one particle per cell. An anti-CR3 antibody (M1/70), that is known to block C3bi binding [2] and that inhibits by ≥95% the number of C3bi-opsonized particles that bind to CR3-transfected COS cells [16], also reduces the number of bound particles to one per cell. We conclude therefore that inhibition of Rap1 in J774 macrophage cells essentially eliminates binding of C3bi-opsonized red blood cells to the αMβ2 complement receptor. TcdB-10463, another *C. difficile* toxin that inhibits Rho, Rac and Cdc42 [20] and has previously been shown to inhibit CR3-mediated phagocytosis [16], did not affect binding of C3bi-opsonized RBC to macrophages (data not shown). Neither Rap1 (N17) nor TcdB-1470 affected the ability of J774 cells to bind IgG-opsonized targets (data not shown).

Figure 2



Rap1 function is sufficient for functional activation of αMβ2 in macrophages and is necessary for PMA-induced CR3-mediated phagocytosis. **(a)** Expression of active R-Ras (V-38) or Rap1 (V12) in J774.A1 cells bypasses the need for cell activation for the acquisition of CR3-dependent phagocytic potential. Resting macrophages were microinjected with biotin dextran (left panels), Rap1 (V12) (centre panels) or R-Ras (V38) (right panels), fed with red blood cells and stained for biotin or Myc expression (top) and red blood cell markers (bottom). Phagocytosed particles appear swollen and lie within vacuoles. The scale bar represents 10 μm. **(b)** Rap1 and R-Ras, but not Ras or RalA, stimulate phagocytosis in macrophages. **(c)** Rap1 but not R-Ras is necessary for PMA-induced αMβ2 activation in macrophages. **(d)** Rap1 controls the avidity of αMβ2 for its ligand. C3bi-opsonized red blood cells (C3bi–RBC) were incubated with non-activated J774.A1 cells that had been microinjected with various GTPase constructs, or pretreated at 37°C with TcdB-1470 (1 pg/ml, 2.5 h) or anti-CR3 M1/70 (25 μg/ml, 10 min, conditions that inhibit by ≥95% the number of C3bi-opsonized particles that bind to CR3-transfected COS cells, see [16]). Control (C) cells are injected with lysine-fixable biotin dextran (10,000 MW, Molecular Probes) and visualized with AMCA-coupled streptavidin (Molecular Probes). In (d), macrophages were incubated for 30 min with opsonized particles, fixed and processed for immunofluorescence as described, and the total number of particles associated with the cells was counted. Data are expressed as mean ± SEM of at least three experiments, in which a minimum of 35 positive cells were counted per experiment.

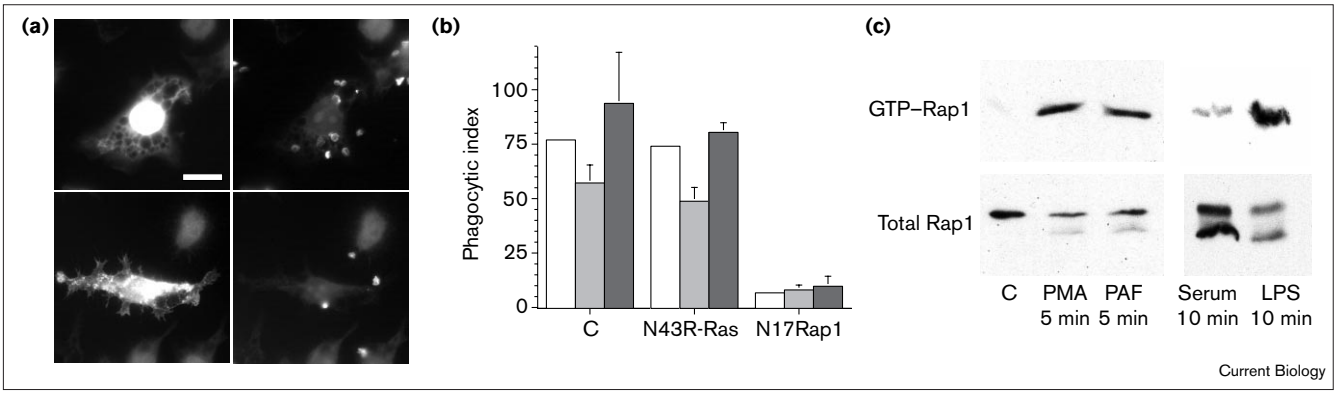
In vivo and *in vitro*, a variety of inflammatory signals can activate β2-dependent functions in cells of the immune system. In agreement with previous reports [21], we find that three distinct agonists, TNF-α, LPS and PAF, induce CR3-mediated phagocytic potential in J774 macrophages. As with PMA, none of these affected surface expression of αMβ2 (data not shown). To determine whether Rap1 also mediates αMβ2 activation by inflammatory agents, dominant-negative constructs were injected into macrophages before addition of the inflammatory agents. Figure 3a,b shows that dominant-negative Rap1, but not dominant-negative R-ras, completely inhibited β2-dependent phagocytosis induced by LPS, TNF-α and PAF. These results argue for a specific role for Rap1 — not R-Ras — in agonist-induced activation of β2 integrins in macrophages. Why active R-ras promotes αMβ2 activation when overexpressed is not clear: it might activate Rap1 or a common R-Ras/Rap1 effector.

Finally, to confirm that each of these agonists is capable of activating endogenous Rap1, we have carried out pull-down assays using a RalGDS–GST fusion protein, to determine the levels of GTP-bound Rap1 in cells (Figure 3c). Whereas control cells do contain detectable levels of GTP-bound Rap1, all three inflammatory mediators induced a significant increase in Rap1 GTP levels (Figure 3c, and data not shown), which preceded the acquisition of phagocytic potential.

Conclusions

In conclusion, we have identified three distinct receptors for inflammatory mediators that induce functional activation of the macrophage integrin, αMβ2, through a Rap1-controlled signal transduction pathway. LPS and TNF-α interact with the Toll/IL-1 and TNFR family of receptors, respectively, which mediate their intracellular effects through adaptor-like proteins such as Myd88, TRAFs and TRADD, whereas PAF acts on a serpentine, G-protein-coupled receptor [22–24]. As numerous Rap1-specific exchange factors (GEFs) have been identified, it remains to be seen whether one or more are involved downstream of these diverse families of receptors [25,26]. The biochemical signals controlled by Rap1 that mediate functional activation of the β2 integrin are unclear, but they appear to be both necessary and sufficient for integrin activation and do not function in these cells by antagonizing Ras [19,27]. Because Ras does not activate αMβ2, the Rap1 effector that mediates this response must be distinct from the known Ras effectors [28]. Similar conclusions have recently been reported, and Rap1 activity has been shown to control the stimulus-induced adhesion, dependent on the cell adhesion molecule LFA-1, of stably transfected cells to surfaces coated with the cell adhesion

Figure 3



Rap1 is necessary for αMβ2 activation mediated by several inflammatory mediators. **(a,b)** Dominant-negative Rap1 (N17) but not R-Ras (N43) prevents activation of αMβ2 by TNF-α, PAF and LPS. In (a) macrophages were injected with biotin dextran (top panels) or Rap1 (N17) (bottom panels), activated with TNF-α and assayed for phagocytosis. Left, injected cells; right, staining for red blood cells. The scale bar represents 10 μm. (b) Macrophages were injected with biotin dextran (C) or Myc-tagged constructs, then 2.5 h later were activated with TNF-α (open bars), PAF (grey bars) or LPS (black bars) and used in phagocytic assays. Results are given as mean ± SEM of at least three experiments, except for TNF-α, where the mean of two experiments is given. **(c)** PMA, LPS and PAF induce GTP loading on Rap1. Top panel, pull-down of GTP-bound Rap1 from lysates of control (C) or activated J774.A1 cells; bottom panel, total lysates (corresponding to 2% of the amount of lysate used in pull-down experiments) were run and probed with anti-Rap1 antibody by western blotting. Results shown in each panel are representative of three independent experiments.

molecule ICAM-1, without affecting β2 surface expression [29,30]. Furthermore, we show that Rap1 acts upstream of the αMβ2 integrin to regulate its ability to bind C3bi-opsonized particles. It is generally thought that three mechanisms can contribute to integrin activation: a change in integrin affinity, by altering its conformation; an increase in avidity through integrin clustering; or a change in the interaction of αMβ2 with the cytoskeleton [31]. There are conflicting reports about the mechanism(s) underlying agonist-induced activation of αMβ2, and exposure of activation-specific epitopes and a role for the actin cytoskeleton have both been proposed [32,33].

We previously showed that Rho acts downstream of CR3 to mediate phagocytosis in macrophages [16] and we show here that Rap1 acts upstream of CR3 to regulate its activity. This nicely demonstrates the cooperation between two distinct small GTPases to promote a coordinated biological response. Finally, it remains to be seen whether Rap1 controls integrin activation in general; in this respect, it is worth noting that Rap1 was first identified as *Krev-1*, a gene able to induce flat revertants in Ras-transformed cells [27]. Nevertheless, in view of its role in controlling β2-integrin activation, both in macrophages, as shown here, and in T cells [29,30], we would expect Rap1 to have a key role in inflammation and immunity *in vivo*.

Materials and methods

R-Ras (V38), Rac (V12) and Rac (N17) [34,35] were subcloned into the mammalian expression vector pRK5myc. Point mutations were introduced by site-directed mutagenesis into wild-type constructs to generate H-Ras (V12), H-Ras (N17), Rap1 (V12), Rap1 (N17), RalA (L72) and R-Ras (N43), which were then subcloned into pRK5myc. pMT2-HA-RapGAP and pMT2-HA-RalA (N28) were provided by Johannes Bos (University of Utrecht, the Netherlands). Purified B toxins, isolated from strains 1470 or 10463 of *C. difficile* (herein referred to as TcdB-1470 and TcdB–10463, respectively), were kind gifts of C. von Eichel-Streiber (Johannes Gutenberg University, Mainz, Germany).

J774.A1 macrophage-like cells (American Type Culture Collection) were grown in DMEM supplemented with 10% heat-inactivated FCS (Sigma). They were treated with bacterial toxins for 2 h at 37°C in serum-free medium (SFM) containing 10 mM HEPES. Cell activation with PMA (Sigma), used at 150 pg/ml for 15 min, recombinant human TNF-α (R&D Systems), used at 50 ng/ml for 30 min, or PAF (Calbiochem), used at 5 nM for 15 min was also performed in HEPES-supplemented SFM. By constrast, LPS (from *Escherichia coli* 055:B5; Sigma) was used at 100 ng/ml for 30 min in DMEM supplemented with 10% heat-inactivated FCS. In all cases, cells were quickly washed in SFM before being used in subsequent assays.

For the analysis of αM surface expression, J774.A1 cells were treated with various agonists or toxins as indicated above, then washed and scraped in cold PBS supplemented with 0.5% BSA, 0.02% azide and excess IgG, and subsequently stained using a rat monoclonal antibody (M1/70, Pharmingen) and FITC-coupled anti-rat IgG (Jackson ImmunoResearch Laboratories). Samples were run on a FACSCalibur (Becton Dickinson) according to the manufacturer's instructions and data were analyzed using the CellQuest software.

Microinjection of J774.A1 cells, the preparation of C3bi-opsonized red blood cells, the phagocytic assay and the staining procedures have been described before [16]. Briefly, cells were transferred to HEPES-containing SFM immediately before injection. The tagged DNA constructs (0.1 mg/ml) were microinjected along with lysine-fixable 10,000 MW biotin dextran (Molecular Probes), at a final concentration of 2.5 mg/ml, into the nucleus of at least 50 cells. Cells were returned to the incubator for 2.5 h, to allow expression of the corresponding proteins, before the phagocytic assay. In some experiments, cells were activated with PMA or inflammatory molecules before the phagocytic

challenge. C3bi-opsonized red blood cells, resuspended in HEPES-buffered SFM, were fed to macrophages for 30 min (target-to-cell ratio = 20) at 37°C. Cells were fixed in 4% paraformaldehyde for 20 min at 4°C and processed for immunofluorescence. Injected cells were visualized using AMCA-coupled streptavidin (Molecular Probes). Phagocytosis was, however, assessed only in the Myc- or haemagglutinin (HA)-positive cells, visualized after immunofluorescent staining using the 9E10 anti-Myc or the 3F10 anti-HA (Boehringer Mannheim) monoclonal antibodies. Phagocytic index is defined as the number of red blood cells internalized per 100 macrophages.

Rap1 pull-downs were conducted essentially as described [36] using 20 μg GST–RalGDS–RBD and 2×10^7 J774.A1 cells per point. Cells were kept in HEPES-supplemented SFM, or stimulated as indicated with PMA (150 ng/ml), PAF (5 nM), serum-supplemented medium or LPS (100 ng/ml) in serum-containing medium. Samples were run on 15% acrylamide SDS–PAGE gels, blotted and probed with a monoclonal anti-Rap1 antibody (Transduction Laboratories) followed by a horseradish peroxidase-conjugated anti-mouse serum (Pierce) and revealed by chemiluminescence (ECL kit, Amersham).

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